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Comparative efficacy of essential oils against *Cutibacterium acnes*: Effect upon strains from phylotypes with different virulence patterns

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ABSTRACT

Objectives: Despite being a commensal of human skin, *Cutibacterium acnes* plays an important role in the pathogenesis of *Acne vulgaris,* with the loss of bacterial phylotype diversity being related to disease progression. This study evaluated differences in the virulence profile of *C. acnes* strains from different phylotypes and investigated the possible phylotype-selective efficacy of essential oils (EOs) from *Thymus* x *citriodorus* (TC), *Thymus mastichina*, and *Cistus ladanifer* compared to *Melaleuca alternifolia* (tea tree), focusing on both planktonic and biofilm growth forms, the latter being related with pathogenesis and treatment resistance.
Methods: One collection strain and seven clinical isolates were classified into phylotypes using multiplex-touchdown PCR. Virulence trait differences across phylotypes were evaluated by studying antibiotic resistance, biofilm formation, porphyrin production, and lipase activity. EOs were tested for minimum inhibitory/bactericidal concentrations (MIC/MBC) and effects on biofilm biomass and metabolic activity.
Results: Strains from phylotype IA_1 were higher biofilm and lipase producers than phylotype II strains. Regarding EO's efficacy, TC EO presented lower planktonic MIC values for all strains compared to the other EOs, presenting a smaller difference in MIC values across phylotypes. TC EO was able to similarly reduce biofilm biomass and metabolic activity in phylotype IA_1 clinical strains, being effective at lower concentrations compared with the remaining EOs.
Conclusions: Not all virulence traits were phylotype-related, highlighting the multifactorial nature of the disease. TC EO showed a relevant anti-acne potential, outperforming tea tree EO (a species with a commercial claim for *Acne vulgaris*) against both planktonic and biofilm growth forms of *C. acnes*.

1. Introduction

Acne vulgaris, commonly known as acne, is an inflammatory skin disease of the pilosebaceous unit that affects millions worldwide [1,2]. This disease has a multifactorial nature, and different hallmarks have been described to be involved in its development, namely: excessive sebum production, epidermal hyperproliferation, increased release of inflammatory mediators, and the classic hypothesis of the *Cutibacterium acnes* bacterium overgrowth [3,4]. *C. acnes* is a gram-positive, rod-shaped anaerobic bacterium, frequently found in sebaceous-rich follicle areas of human skin. This bacterium is typically associated with the progression of acne, still being part of the normal skin microbiota [5,6]. The current hypothesis is that a loss of *C. acnes* phylotype diversity may be involved in the development of acne, rather than bacterium overgrowth [6]. Recent findings report that acne is strongly associated with strains from specific phylotypes, as the IA_1, while others including phylotype II, are more frequently associated with healthy skin [11,42]. Still, the factors that lead to this dysbiosis remain unclear and differences in the expression of putative virulence factors across phylotypes have been investigated to elucidate their distinct involvement in diseased and/or healthy skin [7]. As the pathogenicity of *C. acnes* does not strictly follow Koch's postulates, the role of different virulence factors is hard to pinpoint [5]. Its pathogenicity involves different

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Abbreviations

BHI - Brain Heart Infusion
CL - *Cistus ladanifer*
CLSI - Clinical and Laboratory Standards Institute
DMSO - Dimethyl sulfoxide
DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen
EC_{50} - Half-maximum effective concentration
EOs - Essential oils
EO - Essential oil
EUCAST - European Committee on Antimicrobial Susceptibility Testing
I - Susceptible with increased exposure
MIC - Minimum inhibitory concentration
MBC - Minimum bactericidal concentration
MTT - 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MUO - 4-methylumbelliferyl oleate
OD - Optical density
PCR - Polymerase Chain Reaction
R - Resistant
S - Susceptible
sBHI - Brain Heart Infusion broth supplemented with 5 % of glucose
SDS - Sodium dodecyl sulfate
TC - *Thymus* x *citriodorus*
TE - Tris–ethylenediaminetetraacetic acid
TM - *Thymus mastichina*

mechanisms that allow *C. acnes* to adapt to the pilosebaceous unit environment and trigger host immune responses [8]. Lipase is one of the major metabolic enzymes of *C. acnes*, responsible for the hydrolysis of sebum triglycerides into glycerol and free fatty acids, the last being comedogenic and leading to follicular inflammation [9,10]. Also, the production of porphyrins, intermediate metabolites in the biosynthesis of vital molecules, can stimulate pro-inflammatory responses, contributing to inflammation [11]. The role of biofilms in the pathogenicity of *C. acnes* has been in the spotlight of bacterium-related research since the report of *C. acnes* biofilms in acne lesions, as these lead to increased resistance to antimicrobial agents (due to restricted penetration into the biofilm structure) and are involved in the persistence of the bacterium in the pilosebaceous unit [10,12].

To tackle this multifactorial disease, several drugs have been used including antibiotics [13]. However, these treatments often lead to side effects and bacterial resistance, driving the search for effective and safer alternatives, with *C. acnes* biofilms and specific phylotypes becoming key targets for new therapies [14].

In the last few decades, there has been a growing interest in the use of natural products as an alternative or adjuvant therapies in acne management [15]. Some EOs are already described for their anti-acne potential, as is the case of the EO from *Melaleuca alternifolia* (tea tree EO), and several over-the-counter options containing tea tree EO are available in the market. Other species also present relevance for skin application, including an anti-acne potential, some supported with ethnopharmacological knowledge, such as *Cistus ladanifer* and *Thymus mastichina* [16–18]. Our team has also reported the anti-acne potential of the EO from *Thymus* x *citriodorus* reporting a low minimum inhibitory concentration and an anti-biofilm effect [19].

In this study, we aimed to investigate the differences in virulence trait production among *C. acnes* strains and evaluate potential relations with their respective phylotypes. Based on this, we aimed to compare the effects of EOs from *Thymus mastichina, Thymus* x *citriodorus* and *Cistus ladanifer* with the one from *Melaleuca alternifolia* in inhibiting planktonic and biofilm growth forms of *C. acnes* strains from different phylotypes, to evaluate a possible selective efficacy.

2. Materials and methods

2.1. Chemicals

The microbiological culture media Brain Heart Infusion (BHI) broth and bacteriological Agar type E were supplied by Himedia (L.B.S. Marg, Mumbai, India). Glucose, tetracycline, benzylpenicillin, erythromycin, 4-methylumbelliferyl oleate (MUO), starch and SYTO™ 40 Blue Fluorescent Nucleic Acid Stain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris–ethylenediaminetetraacetic acid (TE) buffer was obtained from Invitrogen (Waltham, Massachusetts, USA). Pore strips used for bacterial collection were purchased from Bioré (Kao, United Kingdom). The anaerobic generating system Anaerocult® A, sodium acetate, coproporphyrin III and clindamycin hydrochloride were supplied by Merck (Darmstadt, Germany). Furazolidone was acquired from TCI Chemicals (VWR, Radnor, Pennsylvania, USA). Yeast extract, peptone and meat extract were purchased from VWR chemicals (VWR, Radnor, Pennsylvania, USA). Sodium hydroxide was acquired from Labkem (Labbox Labware S.L., Barcelon, Spain). Dimethyl sulfoxide (DMSO) and methanol were purchased from Honeywell (Charlotte, North Carolina, USA). The crystal violet stain was acquired from Amresco (Solon, OH, USA). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was supplied by Alfa Aesar (Massachusetts, USA). Hydrochloric acid was acquired from Labsolv (RCI Labscan, Bangkok, Thailand). Ethyl acetate, acetic acid and Tris-HCl were supplied by Fisher Chemicals (Fisher, Chicago, IL, USA). Sodium dodecyl sulfate (SDS), sodium chloride, L-cysteine, hydrogen peroxide and calcium chloride were purchased from PanReac AppliChem (Barcelona, Spain). Primer sets were synthesized by STAB VIDA (Caparica, Portugal) and NZYTaq 2x Green Master Mix and GreenSafe Premium were acquired from NZYtech (Lisboa, Portugal).

2.2. Bacterial strains

C. acnes collection strain DSM1897 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. *C. acnes* isolates were collected from skin microbiota of the face of healthy (no dermatological diseases) volunteers using the pore strip method, as previously described [20]. Briefly, following the manufacturer's instructions, after wetting the region with water, samples were taken from the skin (nose region) of the volunteers using cleansing pore strips. Clean gloves were used for each sampling. After 15 min in contact with the skin, the strips were removed and immediately placed onto BHI agar plates supplemented with 5 % of glucose and 8 μg mL^{-1} of furazolidone. The content of the strips was transferred to the agar plates by rubbing the strips on the surface of the agar, under aseptic conditions. The plates were incubated for 72h, under an anaerobic environment generated with Anaerocult® A, to allow bacterial growth. After incubation, strains with macroscopic morphologic characteristics of *C. acnes*, were isolated and checked for microscopic morphology after Gram straining and tested biochemical characteristics of *C. acnes*, specifically a positive catalase test, using a drop of hydrogen peroxide. Strains that complied with these two tests, were subcultured to ensure purity and viability and were included for identity confirmation.

The study has been approved by the Ethics Committee of University of Beira Interior (CE-UBI-Pj-2022-052) and the participants were asked to sign an informed consent and a small questionnaire on demographical

data and their perception of skin health.

2.3. Identity confirmation of the collected strains

Genomic DNA was extracted from isolated colonies using a standard heat treatment with NaOH, as previously described with some modifications [21,22]. Briefly, one isolated colony was resuspended in 20 μL of lysis buffer (SDS 0.25 %, NaOH 0.05M) and heated for 15 min at 95 °C, on microcentrifuge tubes. After, the tubes were placed on ice and 180 μL of TE buffer was added. The tubes were centrifuged at 1400×*g* in Mini-iSpin® plus (Mini Microcentrifuge, Eppendorf) during 5 min and the upper aqueous phase containing the DNA was transferred into a sterile clean tube and used directly in the polymerase chain reaction (PCR) reaction. Specific primers were used to amplify a region of the *recA* gene of *C. acnes*, as described [22]. The used primer sets were (5′->3′) PR 264: GCAGGCAGAGTTTGACATCC and PAR-2: GCTTCCTCA-TACCACTGGTCATC. The reaction mixtures contained 1 μL of gDNA, 6.25 μl of NZYTaq 2x Green Master Mix, 0.25 μM of each primer, and 4.5 μL of nuclease-free water, in a total of 12.5 μL. Samples were amplified in T100™ Thermal Cycler (Biorad, Hercules, United States of America) using the following conditions: 95 °C for 3 min, 40 cycles of 94 °C for 30s, 56 °C for 30 s, and 72 °C for 30s, with a final elongation step of 72 °C for 7 min. The PCR products were visualized by electrophoresis on 1 % agarose gel with 0.02 μL mL^{-1} of Greensafe.

2.4. Phylotype classification by multiplex-touchdown polymerase chain reaction

Once purity and identity were established, a six-locus multiplex touchdown PCR was performed to reconfirm the identity of the recovered isolates and to classify them in different phylotypes, as previously developed and described by Barnard et al. [23]. The reaction mixtures contained 1 μL of gDNA, 6.25 μl of NZYTaq 2x Green Master Mix, 0.25 μM of each primer, and 4.5 μL of nuclease-free water, in a total of 12.5 μL. The used primer sets are presented in Table 1. Samples were amplified in T100™ Thermal Cycler (Biorad, Hercules, United States of America) using the following conditions: 95 °C for 3 min, 14 cycles of 94 °C for 30s, 68 °C (decreasing incrementally by 0.3 °C per cycle) for 30 s, and 72 °C for 60s, followed by 11 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 60s, with a final elongation step of 72 °C for 10 min. The PCR products were visualized by electrophoresis on 1.5 % agarose gel with 0.02 μL mL^{-1} of Greensafe. The PCR was repeated to confirm the results and the isolates were then categorized into one of the six distinct phylotypes (IA_1, IA_2, IB, IC, II or III) based on their specific amplification profiles, as described in [23].

Table 1
Primers sets used in *C. acnes* phylotyping. Adapted from [23].

Primer		Target	Sequence (5' ->3')	Product size (bps)
PArA-1	All *C. acnes*	16S rRNA	AAGCGTGAGTGACGGTAATGGGTA	677
PArA-2			CCACCATAACGTGCTGGCAACAGT	
PAMp-1	Type IA_1/ IA_2/IC	ATPase	GCGTTGACCAAGTCCGCCGA	494
PAMp-2			GCAAATTCGCACCGCGGAGC	
PAMp-3	Type IA_2/IB	*sodA*	CGGAACCATCAACAAACTCGAA	145
PAMp-4			GAAGAACTCGTCAATCGCAGCA	
PAMp-5	Type IC	Toxin, Fic family	AGGGCGAGGTCCTCTTCTACCAGCG	305
PAMp-6			ACCCTCCAACTGCAACTCTCCGCCT	
PAMp-7	Type II	*atpD*	TCCATCTGGCCGAATACCAGG	351
PAMp-8			TCTTAACGCCGATCCCTCCAT	
PAMp-9	Type III	*recA*	GCGCCCTCAAGTTCTACTCA	225
PAMp-10			CGGATTTGGTGATAATGCCA	

2.5. Virulence evaluation

2.5.1. Biofilm formation capacity

Biofilm formation dynamics were assayed by crystal violet staining of the attached biomass in 96-well microtiter plates after 72h, as previously described, with some modifications [5]. *C. acnes* strains were used to prepare a bacterial pre-inoculum, for 3 days in Brain Heart Infusion broth supplemented with 5 % of glucose (sBHI), under anaerobic conditions. After incubation, a 96-well plate was filled with 100 μL per well of the adjusted inoculum at 0.5 MacFarland and incubated for 72h at 37 °C, under an anaerobic environment. After incubation, the growth of each *C. acnes* isolate was monitored by resuspending the biomass of at least two wells per strain and measuring OD_{600} using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The remaining wells (six per *C. acnes* isolate) were used for biofilm quantification. The supernatant with the planktonic bacteria was removed and the remaining biofilm was fixed by adding 100 μL methanol. After drying, the biofilm was stained with 0.05 % crystal violet for 15 min, the non-bound stain was removed by washing with 200 μL of type I MiliQ water, and the bound crystal violet was dissolved with 150 μL of acetic acid at 33 % (v/v). The OD at 590 nm was measured to quantify the amount of biofilm biomass.

Wells filled with sterile sBHI were included as a sterility control and served as blank for the OD_{600} and OD_{590} measurements. Each isolate was tested in sextuplicate in three independent experiments. The results from different strains were evaluated individually and grouped into the corresponding phylotypes. The biofilm formation capacity was represented as a ratio between OD_{590} and OD_{600} readings and the statistical significance between groups was determined using an unpaired *t*-test with GraphPad Prism V8 software.

2.5.1.1. Biofilm imaging. To elucidate the biofilm's architecture, confocal microscopy was performed. Biofilms were formed as described in "2.5.1. Biofilm formation capacity", using 96 well-black plates with clear bottoms. After a fixation step with 100 μl of methanol, biofilms were strained with 100 μL per well of 20 μM SYTO™ 40 in TE buffer, and incubated in the dark at room temperature according to supplier specifications. Fluorescence from SYTO™ 40 was excited at 405 nm and collected at 486 nm, with a detection wavelength of 423–549 nm. Images were acquired on a LSM710 confocal laser scanning microscope. For each strain, representative *z*-stacks comprising the full thickness of biofilm (30 μm) were captured. The image processing was performed using the software ZEN (ZEISS Microscope Software version 3.10), maintaining the same settings across different images.

2.5.2. Lipase activity

Measurement of lipase activity was evaluated as previously described by [24,25]. Briefly, bacterial pre-inoculums were grown in sBHI for 72 h h under anaerobic conditions. After, the OD was adjusted to 2 MacFarland and 100 μL of each strain was plated in a 96-well plate in quintuplicate and incubated for 24h at 37 °C in the conditions described above. After incubation, the contents of two wells per strain were resuspended and the OD_{600} was determined using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) to evaluate bacterial growth. The plate was centrifuged at 3000×*g* for 20 min and the contents of the remaining wells (50 μl of each) were mixed with 50 μl of a MUO solution at 10 mM prepared in 13 mM Tris-HCl, 0.15 M NaCl, and 1.3 mM $CaCl_2$ (pH 8.0). The mixtures were incubated for 30 min at 37 °C

in the dark. After incubation, enzymatic reactions were terminated by adding 100 μL of 0.1 M sodium citrate (pH 4.2). The levels of 4-methylumbelliferone released by the lipase were measured using a fluorometric microplate reader SpectraMax Gemini EM Microplate Reader (Molecular Devices), at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Controls without MUO for each strain and without lipase (culture medium only) were also included. Lipase activity of each strain was represented as a ratio between Ex_{355nm}/Em_{460nm} and OD_{600} readings. The results from different strains were evaluated individually and grouped onto the correspondent phylotypes and the statistically significant differences between phylotypes were determined using unpaired *t*-test with GraphPad Prism V8 software.

2.5.3. Porphyrin quantification

Porphyrin extraction was performed as previously reported, with minor modifications [26]. Briefly, *C. acnes* strains were cultured in reinforced clostridium broth [10 g L^{-1} meat extract, 10.0 g L^{-1} peptone, 3.0 g L^{-1} yeast extract, 5.0 g L^{-1} D(+) glucose, 1 g L^{-1} starch; 5.0 g L^{-1} sodium chloride, 3.0 g L^{-1} sodium acetate, 0.5 g L^{-1} L-cysteine and 0.5 g L^{-1} bacterial agar] anaerobically without exposure to light at 37 °C during 14 days in order to reach stationary phase. After incubation, 200 μL of bacterial cultures were collected to measure optical density at 600 nm and 500 μL were used to extract porphyrins. To do so, the bacterial cultures were mixed with 250 μL of ethyl acetate/acetic acid (4:1) for 10 s by vortexing, and centrifuged for 5 min at 12,000 rpm. The upper phase was transferred to a new microcentrifuge tube and mixed with 250 μL of 1.5M HCl for 10 s. The mixture was centrifuged for 2 min at 12, 000 rpm, and 200 μL of extracted porphyrins in HCl lower phase were recovered for quantification. Absorbance values were measured at 400 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) and converted to concentration, using a standard curve of coproporphyrin III, prepared in 1.5M HCl, at a concentration range from 0.3 μM to 10 μM ($R^2 = 0.998$; data not shown), as described by Gabler et al. [27]. The porphyrin production was represented as a ratio between the porphyrin concentration (μM), obtained from the coproporphyrin III standard curve, and OD_{600} readings representative of bacterial growth. The results from different strains were evaluated individually and grouped into the corresponding phylotypes. The statistically significant difference between the two groups was determined using unpaired *t*-test with GraphPad Prism V8 software.

2.5.4. Antibiotic susceptibility profile

The antibiotic susceptibility testing for tetracycline, benzylpenicillin, clindamycin and erythromycin was performed using the Clinical and Laboratory Standards Institute M11-A6 microdilution method [28,29]. Briefly, a 0.5 MacFarland suspension was prepared from bacterial cultures using sterile NaCl solution at 0.85 % (w/v) and proper dilutions were made with sBHI. The bacterial suspensions were exposed to serial dilutions of antibiotics, priorly diluted in sterile H_2O (tetracycline, benzylpenicillin, clindamycin) or DMSO (erythromycin). After 72h of incubation under an anaerobic environment generated with Anaerocult® A, the minimum inhibitory concentration (MIC) was defined for each antibiotic, as the lowest concentration that caused a complete absence of microbial growth, determined by visual inspection of the plates. Also, due to the inter-operator variability associated with visual inspection methods, absorbance was measured at 600 nm to confirm the visual readings [30].

Concentration ranges were selected based on susceptibility breakpoints described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for clindamycin (max. 8 μg mL^{-1}) and benzylpenicillin (max. 0.5 μg mL^{-1}). Tetracycline and erythromycin, antibiotics with clinical relevance in acne disease but without described susceptibility breakpoints, were tested in the same concentration ranges for clindamycin and benzylpenicillin, respectively. The strains were classified as susceptible (S), resistant (R) or susceptible with increased exposure (I) to each antibiotic, based on updated MIC breakpoints.

2.6. Evaluation of antimicrobial activity of essential oils

2.6.1. Essential oils

All EOs were acquired from Portuguese aromatic and medicinal plant producers. *Thymus mastichina* EO was obtained from Planalto Dourado™, *Thymus* x *citriodorus* and *Melaleuca alternifolia* EOs were obtained from Ervitas Catitas™ and *Cistus ladanifer* EO was acquired from Proentia®. Plants were produced under organic farming, as certified by PT-BIO-04 (for TM, TC and TT) and PT-BIO-03 (for CL). Information about the tested EOs and their chemical composition is provided in Table 2.

2.6.2. Minimum inhibitory concentration determination

The evaluation of antimicrobial activity of EOs was performed as described in "2.5.4. Antibiotic susceptibility profile" section using the Clinical and Laboratory Standards Institute M11-A6 microdilution method [28,29]. Sterile DMSO was used as a surfactant to allow the dispersion of EOs in the culture media. EOs were firstly dissolved in sterile DMSO (50 % v/v) and, from those, working solutions were prepared in sBHI [32]. The dilutions were prepared by vigorous vortexing and were additionally mixed by pipetting before being added to each microplate well. EOs were tested at a concentration ranging from 0.01 % (v/v) up to 2 % (v/v). The adjusted microbial suspensions were exposed to serial dilutions EOs resulting in a 2-fold dilution of both the EOs and the microbial suspensions. After 72h of incubation, the MIC was defined as the first EO concentration with an absence of microbial growth, determined by visual inspection, with confirmation by spectrophotometric readings at 600 nm, as described in section "2.5.4. Antibiotic susceptibility profile". In addition to growth control (bacteria in sBHI) and sterility control (un-inoculated medium), a surfactant control (DMSO) in the maximum tested concentration was also included to discard its influence on bacterial growth. MIC determinations were carried out in, at least, triplicates, in three independent experiments.

2.6.3. Minimum bactericidal concentration determination and MBC:MIC ratio

Minimum bactericidal concentrations (MBC) were determined by inoculating 5 μl of the content of each well with no bacterial growth visually assessed, on sBHI agar, and incubating them for an additional 72h period, as previously described [31]. MBC was defined as the first concentration where no bacterial colonies were present after incubation. MBC determinations were carried out in, at least, triplicates, in three independent experiments.

MBC and MIC values were used to calculate the MBC:MIC ratio to elucidate the type of antibacterial effect. If the ratio MBC:MIC ≤ 4, the effect was considered bactericidal; if the ratio MBC:MIC >4, the effect was defined as bacteriostatic, as previously described [33–35].

2.6.4. Effect of essential oils upon C. acnes preformed biofilms

The effect of the different EOs on biofilm growth forms of *C. acnes* strains was further determined, using two different protocols, previously described [19,36]. Bacterial pre-inoculums were grown for three days in sBHI under anaerobic conditions, and used to prepare bacterial suspensions. The bacterial suspensions were diluted in sBHI to achieve 10^6 CFU mL^{-1} and plated onto 96-well flat-bottom microdilution plates. The plates were incubated for 72h to allow biofilm formation. After incubation, culture media and planktonic cells were removed, and the dilutions of the different EOs prepared in sBHI, were added to the wells and incubated for an additional 72h period. After incubation, biofilms were washed once with PBS 1X to remove the planktonic phase and were treated depending on the intended output reading.

For biomass quantification, after washing, biofilms were fixed using 100 μL of methanol. After that, the methanol was discarded and the plates were allowed to air dry. The adherent phase was then stained with 0.05 % crystal violet for 20 min. After staining, the plates were washed with 200 μL of water to remove the non-bound stain and the bound crystal violet was dissolved with 150 μL of acetic acid at 33 % (v/v). The

Table 2
Information on source and chemical composition (≥5 % of relative abundance) of the tested EOs.

EO	Plant Species	Common name	Source	Batch	Major compounds	Ref.
TC	*Thymus* × *citriodorus* (Pers.) Schreb.	Lemon thyme	Ervitas Catitas™	07TC20	Geraniol (27.5 %), 1,8-cineole (16.3 %), thymol (9.2 %)	[19]
TM	*Thymus mastichina* L. (L.)	Mastic thyme	Planalto Dourado™	TM010719	1,8-Cineole (43.5 %), *p*-cymene (16.8 %), β-pinene (5 %)	[31]
CL	*Cistus ladanifer* L.	Rockrose	Proentia®	6	α-Pinene (50 %), camphene (10.1 %),	[31]
TT	*Melaleuca alternifolia* (Maiden & Betche) Cheel	Tea tree	Ervitas Catitas™	08MA20	1,8-Cineole (40.7 %), terpinen-4-ol (18.9 %), γ-terpinene (9.4 %), limonene (5.2 %)	This work[a]

[a] Information on the chemical composition obtained by Gas Chromatography coupled to Mass Spectrometry (GC-MS) for component identification, and by Gas Chromatography with Flame Ionization Detector (GC-FID) for component quantification, was obtained from the EO analysis datasheet provided by Ervitas Catitas™.

optical density was measured at 590 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad).

For the metabolic activity evaluation, after washing, 100 μL of a MTT solution at 0.1 mg mL^{-1} prepared in sBHI was added to each well, and incubated in the dark at 37 °C for 2h. Then, the MTT solution was removed, and the formed formazan crystals were dissolved with 100 μL of DMSO. Absorbance was measured at 570 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad).

EOs were tested at concentrations ranging from one-fourth, half, once, twice to four times the obtained planktonic MIC value for each *C. acnes* isolate. Negative controls (in the absence of EOs) corresponding to the highest biofilm biomass or metabolic activity, and sterility controls (non-inoculated medium) were included in both protocols. Each condition was tested in quadruplicate, in, at least, three independent experiments. The effect of EOs on biofilm biomass or metabolism was normalized to the control and presented as a percentage of biofilm biomass or metabolic activity. The results from different strains were grouped into the correspondent phylotypes. Half-maximum effective concentration (EC_{50}) was estimated for each output by logistic regression and EC_{50} values were compared for statistically significant differences ($p < 0.05$) using the comparison of fits, with GraphPad Prism version 8 for Windows.

3. Results

3.1. Strain's phylotyping and virulence assessment

3.1.1. Classification into phylotypes

From the collected strains, five were classified as belonging to phylotype IA_1, and two belonged to phylotype II. Only one *C. acnes* strain was recovered per volunteer. The collection type strain DSM1897 was classified into IA_1 phylotype. The source and classification of the strains included in this study, obtained by multiplex-touchdown PCR (Fig. 1), is disclosed in Table 3.

3.1.2. Biofilm formation capacity

The strains were assessed regarding their biofilm formation capacity, by measuring total biofilm biomass normalized by bacterial growth (Fig. 2). As presented in Fig. 2a, individual strains belonging to phylotype IA_1 produced higher levels of biomass compared with individual strains belonging to phylotype II. DSM strain (collection strain) was an exception to this pattern, showing similar results to type II strains regarding biofilm biomass. The difference between biofilm formation capacity among clinical strains from the distinct phylotypes was statistically significant, as presented in Fig. 2b. The difference in biomass production from collection and clinical strains belonging to the same phylotype (IA_1) was also statistically significant.

Optical sections acquired through 3D reconstructions of biofilm structures representative of clinical strains from each phylotype and the collection strain, evidencing a difference in their structure, are represented in Fig. 3. Clinical strains from phylotype IA_1 presented a more complex architecture and well-structured three-dimensional biofilms with distinct pillars and channels. Contrarily, biofilms from clinical strains from phylotype II present a low-structured three-dimensional morphology, showing a flatter layer of stacked cells. The biofilm from the collection type strain (DSM), despite being less dense, presented a microscopic structure closer to the ones from phylotype IA_1 strains, as denoted by Fig. 3. Optical sections of 3D reconstructions of biofilms for all strains under test are presented in supplementary material (Fig. S1).

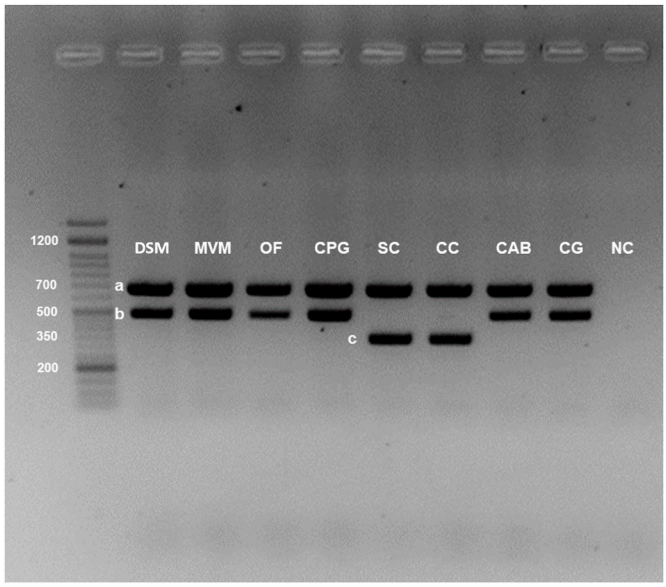

Fig. 1. Multiplex PCR analysis of *C. acnes* strains, representing different phylotypes. Lane 1 - molecular size marker (Ladder VI from Nzytech, Portugal). Lane 2 – Collection type strain of *C. acnes*, belonging to the IA_1 phylotype. Lanes 3,4,5,8,9 – Clinical *C. acnes* strains, recovered from the skin of volunteers, belonging to the IA_1 phylotype. Lanes 6,7 - Clinical *C. acnes strains*, recovered from the skin of volunteers, belonging to the II phylotype. Lane 10 – No template (negative) control. Gene amplicons: a – 16S rRNA (677bps); b – ATPase (494 bps); c – *atpD* (351bps).

Table 3
C. acnes strain panel included in the study, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (collection strain) or recovered from the skin of volunteers (clinical strains) and their classification into different phylotypes.

Isolate	Acronym	Source	Phylotype
DSM1897	DSM	Collection	IA_1
MVM_24022022_1	MVM	Clinical	IA_1
OF_22092021_3	OF	Clinical	IA_1
CPG_22092021_1	CPG	Clinical	IA_1
SC_09092021_2.1	SC	Clinical	II
CC_24022022_1	CC	Clinical	II
CAB_21012022_1	CAB	Clinical	IA_1
CG_22092021_3	CG	Clinical	IA_1

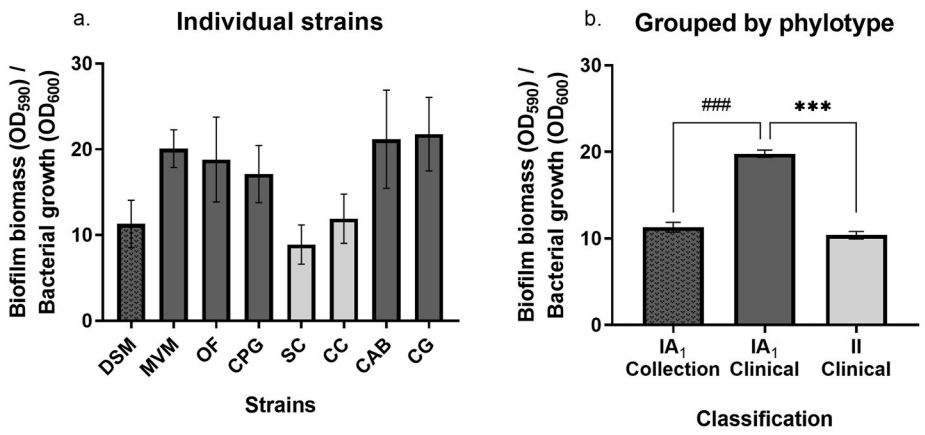

Fig. 2. Biofilm formation capacity of *C. acnes* isolates. (a.) Show the biofilm formation of individualized *C. acnes* strains, quantified after 72h and (b.) show the biofilm formation of *C. acnes* strains grouped into different phylotypes, specifically IA_1 and II. Biofilm formation capacity is represented as a ratio of OD_{590}/OD_{600} measurements. ***represents a statistically significant difference between phylogroups in the clinical strains as determined by a *p*-value<0.001, obtained with unpaired *t*-test. ###represents a statistically significant difference between the collection strain and clinical strains of a specific phylogroup as determined by a *p*-value<0.001, obtained with unpaired *t*-test.

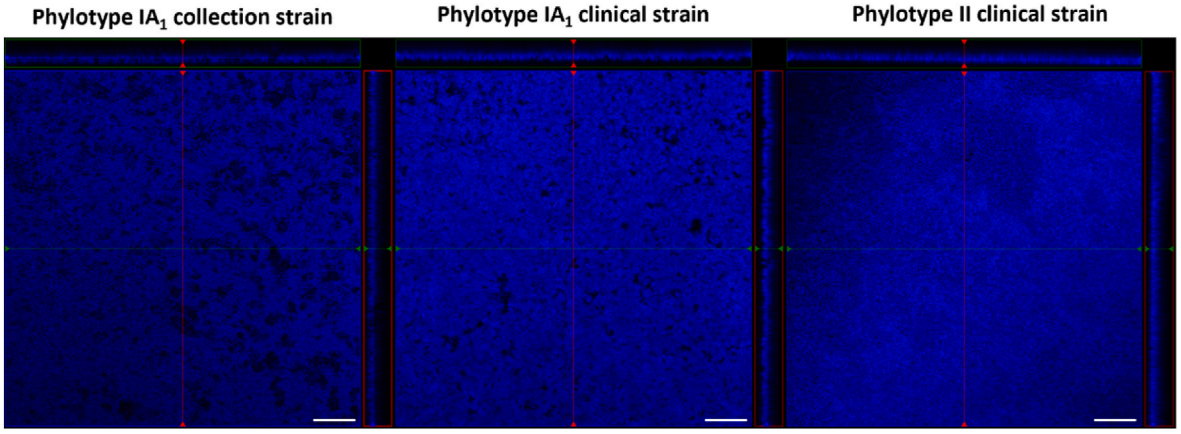

Fig. 3. Orthogonal images from confocal laser scanning microscopy of *C. acnes* biofilms showing structural differences in biofilms formed by *C. acnes* strains from different phylotypes. Images show fluorescent microscopy images of SYTO™ 40 stained *C. acnes* biofilms as horizontal and vertical projections. Large panels represent optical sections through the acquired three-dimensional data at the marked z position. The type of strain (collection or clinical) used as representative of each phylotype is indicated on top of each image. The scale bar represents 50 μm.

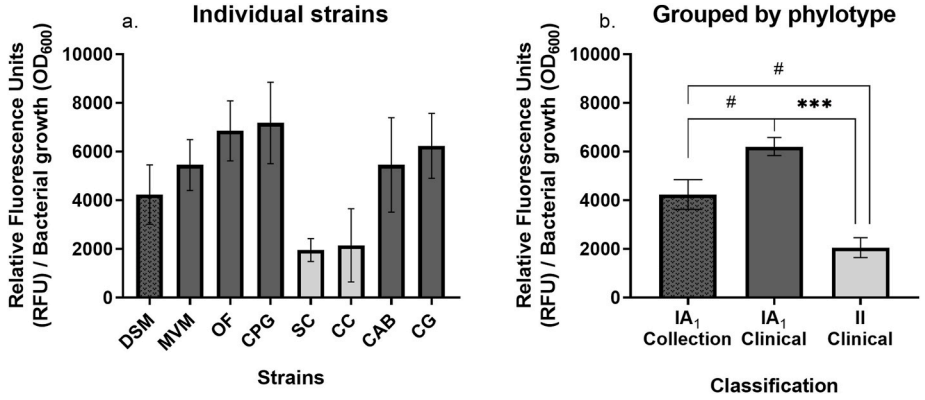

Fig. 4. Lipase activity of *C. acnes* isolates. (a.) Show the lipase activity of individualized *C. acnes* strains, quantified by the fluorescence levels produced by 4-methylumbelliferone (b.) Show the lipase activity *C. acnes* strains grouped into different phylotypes, specifically IA_1 and II. ***represents a statistically significant difference of lipase activity between phylogroups in the clinical strains as determined by a *p*-value<0.001, obtained with unpaired *t*-test, and #represents a statistically significant difference between the collection strain and clinical strains of a specific phylogroup as determined by *p*-value<0.05.

3.1.3. Lipase activity

Results from the lipase activity of each *C. acnes* isolate were determined by the lipase-catalyzed cleavage of 4-MUO that produces the fluorescent product 4-methylumbelliferone, in a proportion related to the enzyme activity. Lipase activity results, normalized by the amount of bacterial growth, are presented in Fig. 4a for individual strains, and in Fig. 4b for collection and clinical strains grouped by phylotype. As presented in Fig. 4a., strains belonging to phylotype IA_1 presented a higher lipase activity than the strains belonging to phylotype II, and this difference was statistically significant, as denoted by Fig. 4b. The collection strain from phylotype IA_1, produced lower lipase levels compared to clinical strains from the same phylotype, still producing higher levels than the ones from type II strains.

3.1.4. Porphyrin quantification

The levels of porphyrins produced by *C. acnes* strains on stationary phase were determined using a coproporphyrin III standard curve and the values, normalized by bacterial growth, are presented in Fig. 5. As expressed in Fig. 5a., the production of porphyrin appears to be strain-specific, as different strains belonging to the same phylotype produced distinct levels of porphyrin. The strain with the higher production was the CAB clinical strain, followed by the collection strain DSM. When grouped into different phylotypes, despite a trend for higher production in type IA_1 clinical strains, the results were not statistically significant, as represented in Fig. 5b. A statistically significant difference was present between the phylotype IA_1 collection strain and type II clinical strains.

3.1.5. Antibiotic resistance

Antimicrobial susceptibility of *C. acnes* strains to antibiotics with clinical relevance in acne disease was evaluated and visual MIC values are disclosed in Table 4.

Overall, all strains presented lower MIC values than the susceptibility breakpoints described for clindamycin and benzylpenicillin in EUCAST guidelines, specifically $\leq$0.25 μg mL^{-1} for clindamycin and $\leq$0.06 μg mL^{-1} for benzylpenicillin, thus being considered susceptible to those antibiotics. Small variations among strains were found for benzylpenicillin, with the DSM strain presenting the lower MIC value for this antibiotic. No major differences between the two phylotypes were found.

For tetracycline and erythromycin, MIC values for phylotype II strains were lower (half) than for phylotype IA_1 strains, being 0.25 μg mL^{-1} for tetracycline and 0.01 μg mL^{-1} for erythromycin.

Table 4
Visual MIC values for different antibiotic controls against tested strains. The susceptibility profile of the tested bacteria to each antibiotic is represented in brackets next to the obtained MIC value.

	Benzylpenicillin MIC (μg mL^{-1})	Clindamycin MIC (μg mL^{-1})	Erythromycin MIC[a] (μg mL^{-1})	Tetracycline MIC[a] (μg mL^{-1})
DSM	0.01 (S)	0.03 (S)	0.03	0.50
MVM	0.03 (S)	0.03 (S)	0.03	0.50
OF	0.03 (S)	0.03 (S)	0.03	0.50
CPG	0.03 (S)	0.03 (S)	0.03	0.50
SC	0.06 (S)	0.03 (S)	0.01	0.25
CC	0.03 (S)	0.03 (S)	0.01	0.25
CAB	0.03 (S)	0.03 (S)	0.03	0.50
CG	0.03 (S)	0.03 (S)	0.03	0.50

S – Susceptible.
[a] *C. acnes* susceptibility profile is not available in EUCAST guidelines.

3.2. Evaluation of the essential oils' efficacy against C. acnes planktonic and biofilm growth forms

3.2.1. Antimicrobial activity of EOs

Overall, all the tested EOs inhibited *C. acnes* growth, as visual MIC values were obtained for all strains, at the tested concentration range. The obtained MIC and MBC values are presented in Table 5. The MIC values obtained for strains among each phylogroup are schematically represented in Fig. 6.

The MIC values obtained for TC EO were the lowest of the four EOs

Table 5
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) obtained for *C. acnes* strains, with the tested EOs.

Strain	*Thymus* x *citriodorus*		*Thymus mastichina*		*Cistus ladanifer*		*Melaleuca alternifolia*	
	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)	MIC (% v/v)	MBC (% v/v)
DSM	0.06	0.12	1.00	2.00	0.25	0.50	0.25	0.50
MVM	0.03	0.06	1.00	>2.00	0.50	1.00	0.50	1.00
OF	0.06	0.12	1.00	2.00	0.25	1.00	0.25	0.50
CPG	0.03	0.06	0.50	2.00	0.06	0.50	0.25	1.00
SC	0.03	0.06	0.50	2.00	0.06	0.25	0.25	1.00
CC	0.03	0.06	0.25	0.50	0.06	0.50	0.12	1.00
CAB	0.06	0.12	0.50	1.00	0.125	0.25	0.50	1.00
CG	0.03	0.06	0.50	1.00	0.125	1.00	0.50	1.00

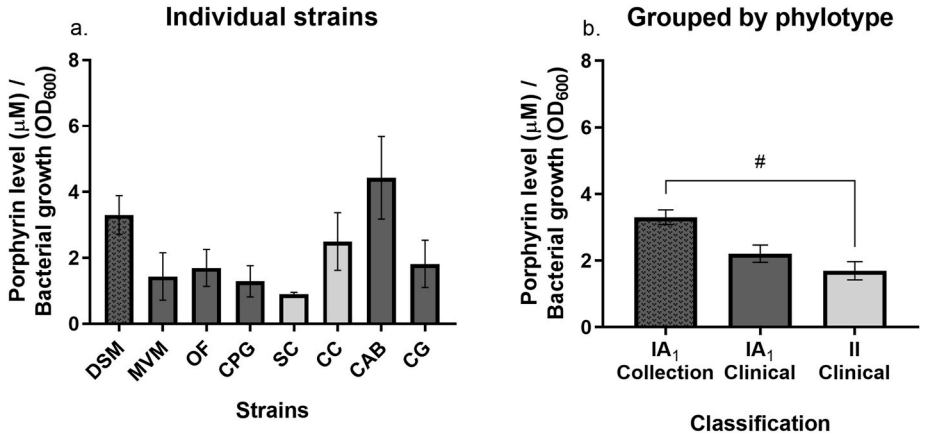

Fig. 5. Porphyrin production by *C. acnes* isolates, represented by porphyrin levels (μM) normalized by bacterial growth (OD_{600} readings). (a.) Show the porphyrin levels produced by individualized *C. acnes* strains and (b.) Show the porphyrin production by *C. acnes* strains grouped into different phylotypes, specifically IA_1 and II. #represents a statistically significant difference between the collection strain, and clinical strains of a specific phylogroup as determined by p-value$<$0.05. No statistically significant difference was found between phylogroups of the clinical strains.

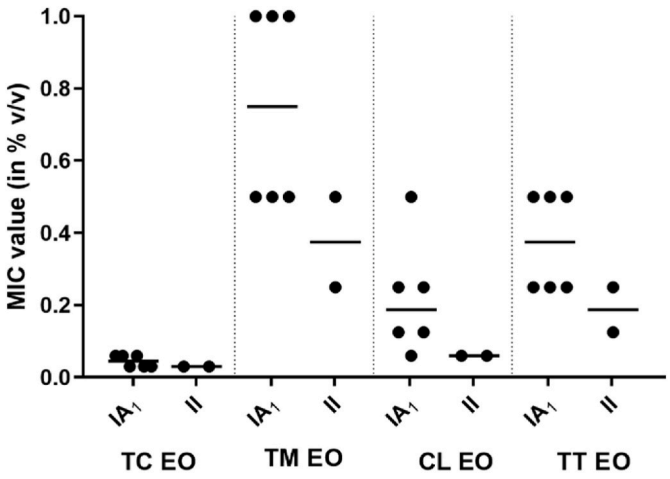

Fig. 6. Antimicrobial activity, represented by minimum inhibitory concentration (MIC), of *Thymus* x *citriodorus* EO (TC EO), *Thymus mastichina* EO (TM EO), *Cistus ladanifer* (CL EO) and *Melaleuca alternifolia* EO (TT EO), for each *C. acnes* strain, represented by individual dots, grouped into different phylotypes. The horizontal line represents the median from the MIC values in each group. No statistically significant differences were found between MIC values for IA_1 and II phylotypes by unpaired *t*-test.

and ranged from 0.03 % (v/v) to 0.06 % (v/v). CL EO presented the second lower values ranging from 0.06 % (v/v) to 0.5 % (v/v). For TM EO and TT EO the MIC values were higher, varying from 0.125 % (v/v) to 0.5 % (v/v) and from 0.25 % (v/v) to 1 % (v/v) for all strains, respectively. Comparing the effect of TC (the EO with better results in terms of efficacy) with the other EOs, MICs for TC EO were 4–16 times lower than TT EO, 2–16 and 4–33 times lower than CL and TM, respectively.

Regarding the effect of the EOs on strains belonging to different phylotypes, overall, the strains belonging to phylotype II were more susceptible to the effect of the EOs, being this difference non statistically significant. Noteworthy, the difference between type IA_1 and phylotype II MIC values was lower for TC EO, as denoted by Fig. 6.

The type of antibacterial activity was estimated using the MBC:MIC ratio as presented in Fig. 7. Considering the type of antimicrobial effect

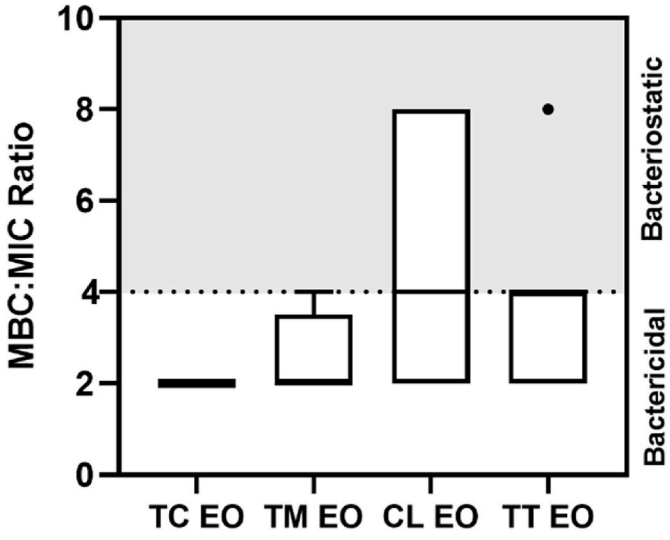

Fig. 7. Box-plot of the MBC:MIC ratios for the different EOs. The center line denotes the median value, and the whiskers mark the minimum and maximum values. Values beyond these upper and lower bounds were considered outliers, and are marked with dots. MBC:MIC ratio ≤4 indicates a bactericidal effect and MBC:MIC ratio >4 defines a bacteriostatic effect.

of the different EOs, TC, TM and TT EOs, presented a bactericidal effect as the MBC:MIC ratio was ≤4. Specifically for TC EO, MBC was twice the MIC value for all strains under test, highlighting its higher efficacy. For CL EO, the effect was strain-dependent, still showing a bacteriostatic tendency.

3.2.2. *Anti-biofilm activity of essential oils*

The effects of the EOs on the total biofilm biomass and on the metabolic activity of the cells within the biofilms were evaluated, testing a range of concentrations ranging from ¼ to 4 times the planktonic MICs. These MIC proportions represent different concentration values as individual strains present a specific MIC value for each EO. The EC_{50} estimated for each strain are presented in Table 6. The graphical representation of the EO's effect on *C. acnes* strains grouped into different phylotypes is represented in Fig. 8.

TC EO was able to impair both biofilm biomass and metabolic activity with lower concentrations when compared with TM, CL and TT EOs, as presented by the overall lower EC_{50} values, and lower conservative means. With this EO, for all clinical strains belonging to phylotype IA_1, the concentrations necessary to impair both the biomass and the metabolic activity were similar (no statistical difference). The same profile was not verified for the remaining EOs, where some strains required higher concentrations to impair one of the two outputs compared to the other, with the majority of the phylotype IA_1 strains requiring a higher concentration to disrupt biomass than to impair metabolic activity. Contrarily, for phylotype II clinical strains, the EOs were more effective in disrupting biofilm biomass, presenting statistically significant lower EC_{50} values, than in impairing cellular metabolism. This pattern was also present for the collection strain, with the four EOs. When comparing the two phylotypes, EOs were able to disrupt the biofilm biomass of phylotype II strains with lower concentrations when compared to phylotype IA_1 strains, as denoted by lower EC_{50} conservative means.

4. Discussion

Acne vulgaris is a multifactorial disease of the pilosebaceous unit and the overgrowth of *C. acnes* has been described as a classic hallmark of this disease [14]. However, this hypothesis has evolved, as this bacterium is also involved in the maintenance of healthy skin. New findings suggest that a loss of *C. acnes* diversity, particularly of its phylotypes, is associated with acne development [6,23,37–40]. *C. acnes* isolates can be classified into distinct phylotypes that display differences in the production of putative virulence traits [23]. Despite being difficult to pinpoint, virulence traits appear to have different involvements in the disease, from bacterial protection and persistence, adaptation to the pilosebaceous unit microenvironment, to an increased host immune response [8]. Studies have reported that strains from specific phylotypes present a more virulent profile compared with other phylotypes [38,41,42]. Additionally, acne is reported to be associated with the dominance of specific phylotypes, as the IA_1 phylotype, while others are more frequently associated with healthy skin [11,39]. While these may lead to a cause-effect rationale, similar expressions of virulence traits were found in strains from phylotype IA_1 collected from diseased compared to healthy skin. Overall, these divergent findings highlight that acne development likely involves host and environmental factors and that a selective pressure on certain *C. acnes* strains enhancing their pathogenicity, could be involved [38,39].

In this work, we investigated differences in the phenotypical expression of virulence traits across *C. acnes* strains from different phylotypes, typically associated with acne and/or healthy skin. We included virulence factors with different involvements in the disease, to provide an overall characterization of the strain's virulence and to elucidate a possible association with different phylotypes. The majority of the collected strains belonged to IA_1 phylotype, which was expected, as it is the most prevalent phylotype also in healthy skin [43]. The fact

Table 6
Effect of the tested EOs on individual strains, represented by the estimated half-maximum effective concentration (EC_{50}) for the effect on biofilm biomass and metabolic activity. Bold values represent a statistically significant difference ($p < 0.05$) between EC_{50} for biomass and metabolic activity values in a specific strain.

C. acnes strain	TC EO % (v/v)		TM EO % (v/v)		CL EO % (v/v)		TT EO % (v/v)	
	Biomass EC_{50}	Metabolism EC_{50}	Biomass EC_{50}	Metabolism EC_{50}	Biomass EC_{50}	Metabolism EC_{50}	Biomass EC_{50}	Metabolism EC_{50}
DSM	**0.062**	**0.154**	**0.752**	**2.428**	**0.160**	**0.938**	**0.476**	**1.066**
MVM	0.088	0.095	**0.356**	**0.636**	0.614	0.483	0.662	0.814
OF	0.083	0.092	**1.770**	**0.643**	**0.418**	**0.295**	**0.551**	**0.404**
CPG	0.106	0.112	2.000	1.552	>0.250[a]	>0.250[a]	0.859	0.928
CAB	0.210	0.147	**>2.000**[a]	**1.246**	>0.500	>0.500	**1.475**	**0.913**
CG	0.107	0.109	**>2.000**[a]	**1.171**	**>0.500**	**0.408**	1.760	1.641
Conservative mean[b]	0.109	0.118	>1.480	1.279	>0.407	>0.479	0.964	0.961
SC	**0.094**	**>0.125**[a]	**1.414**	**>2.000**[a]	**0.173**	**>0.250**[a]	>1.000[a]	>1.000[a]
CC	**0.044**	**0.095**	**0.123**	**0.256**	**0.083**	**>0.250**[a]	**0.199**	**>0.500**[a]
Conservative mean[b]	0.069	>0.110	0.769	>1.128	0.128	>0.250	>0.600	>0.750

[a] The estimated EC_{50} value is out of the tested concentration range (>4 times the planktonic MIC).
[b] The conservative mean was estimated considering the value of 4MIC in strains for which the EC_{50} was above the highest concentration tested.

that the volunteers presented no signs of disease at sampling time highlights the role of host-related factors in the development of the disease, as some of these strains phenotypically expressed higher levels of putative virulence traits [39]. We found that phylotype IA_1 strains were stronger biofilm formers, and their biofilms presented a more complex three-dimensional structure, corroborating previously described findings [5]. Cavallo et al. also found that strains from IA_1 phylotype were stronger biofilm formers compared to strains from phylotype II. The authors reported that the strain phylotype had a higher impact on biomass production rather than the isolation site, as strains collected from healthy skin or acne lesions produced similar biomass [38]. This was also corroborated by other authors [5]. These findings are aligned with our results, as despite our strains being collected from healthy individuals at sampling time, they presented a high biofilm formation capacity. It also highlights the importance of characterizing strains collected from different skin conditions, including healthy skin, to infer the role of phylotypes in disease development. One hypothesis is that, in acne patients, a hyperseborrheic environment driven by host factors, may favour biofilm-producing strains, giving certain phylotypes a selective advantage and contributing to acne's inflammatory state [38]. Biofilm-related genes, such as *AcsA* and *RcsB*, found in IA_1 but not IB/II, may explain the link between phylotype and biofilm formation, rather than the isolation site [38]. These hypotheses are also supported by data that show no genetic differences between phylotype IA_1 strains obtained from healthy individuals and acne patients, that could explain the association with the different skin statuses [44].

We also evaluated lipase activity in the different phylotypes, an extracellular enzyme that degrades triglycerides from sebum, providing energy to the bacteria [8]. To the host, excessive lipase products, the free short-chain fatty acids, are known promotors of inflammation [45]. In this work, we found that type IA_1 strains had a higher lipase activity when compared with phylotype II strains, corroborating previously reported findings [46]. Studies of genome sequences for phylotype II strains have identified insertions and deletions in putative lipase genes compared to phylotype IA_1 strains, which may help to explain their reduced lipase activity [47]. The fact that our phylotype IA_1 strains produced higher lipase levels, despite being collected from healthy skin, supports the hypothesis that the obstruction of the pilosebaceous unit, caused by an increase in keratinocyte turnover and sebum production, could promote the selective proliferation of these strains that better exploit the lipid-rich microenvironments of the pilosebaceous unit, through high lipase levels, compared to other phylotypes [38].

Considering other virulence factors, and despite it remains to be determined by what mechanisms *C. acnes* utilizes porphyrins, their production and secretion to the perifollicular area have been related to an inflammatory state [48,49]. In this work, despite the trend for higher porphyrin production in type IA_1 strains, no significant relation was found between phylotype and porphyrin levels [49]. Other authors have reported different results. Johnson et al. reported a higher average porphyrin level produced by acne-associated clade IA-2 (correspondent to IA_1 phylotype) when compared with health-associated type II strains [50]. Barnard et al. also reported similar findings [49]. Despite agreeing that a small sample size could account for this conclusion, the high porphyrin levels produced by one of the phylotype II strains led us to hypothesize that this virulence factor could follow a strain-specific pattern. Others have hypothesized that the production of this virulent trait *in vitro* could be independent of their phylotype and that the same strain, *in vivo*, could behave differently depending on their host [51].

Regarding susceptibility to clinically relevant antibiotics, we did not find significant differences among phylotypes, and all strains were susceptible to the tested antibiotics. Antibiotics, topic or systemically, are typically used as first-line therapeutics for acne, depending on disease severity. However, recolonization frequently occurs within a few weeks, providing limited clinical efficacy [52]. Cavallo et al. related the high tolerance rates to antibiotics to the presence of biofilm structures [38]. This highlights the importance of evaluating the effect of therapeutics in bacterial biofilms when aiming for an anti-acne application, considering their part in treatment inefficacy.

Based on the above-discussed premises, and considering the strain's classification and the overall higher virulence obtained for phylotype IA_1 strains, we tested the efficacy of different EOs to clarify if they could exert a selective effect on strains from this phylotype, considering that new antimicrobial treatments should, ideally, target specific lineages, leaving beneficial strains intact or minimizing further dysbiosis [39,53]. We focused on EOs effect on planktonic and biofilm growth forms, as the last has been directly associated with treatment failure and bacterial persistence on the pilosebaceous unit. The target selection was additionally driven by recent perspectives for future acne management that focus on targeting specific subpopulations (phylotypes) of *C. acnes*, along with a focus on the effect on their biofilms [14,54]. Also, the impact of assessing other virulence traits, such as lipase, as targets in the context of treatment is still elusive [55].

Several EOs have been studied regarding their anti-acne potential, with the EO from *Melaleuca alternifolia* (tea tree) gaining major relevance [19,31,56]. TT EO has a high commercial value in anti-acne products and has been reported as the second most commonly used topical treatment, following benzoyl peroxide [57]. Thus, we compared the activity of TT EO with TC EO, previously reported by our team as denoting anti-acne potential, and with TM EO, and CL EO, two autochthonous Portuguese species with reported relevance for skin application and anti-acne potential [19,31].

TC was the most effective EO in inhibiting bacterial growth, with MICs ranging 0.03 % (v/v) to 0.06 % (v/v), followed by CL. TM and TT were less active, presenting higher MICs. Considering their antibacterial properties, TC, TM, and TT showed bactericidal effects, while CL appears bacteriostatic based on their MBC:MIC ratios. The antibacterial

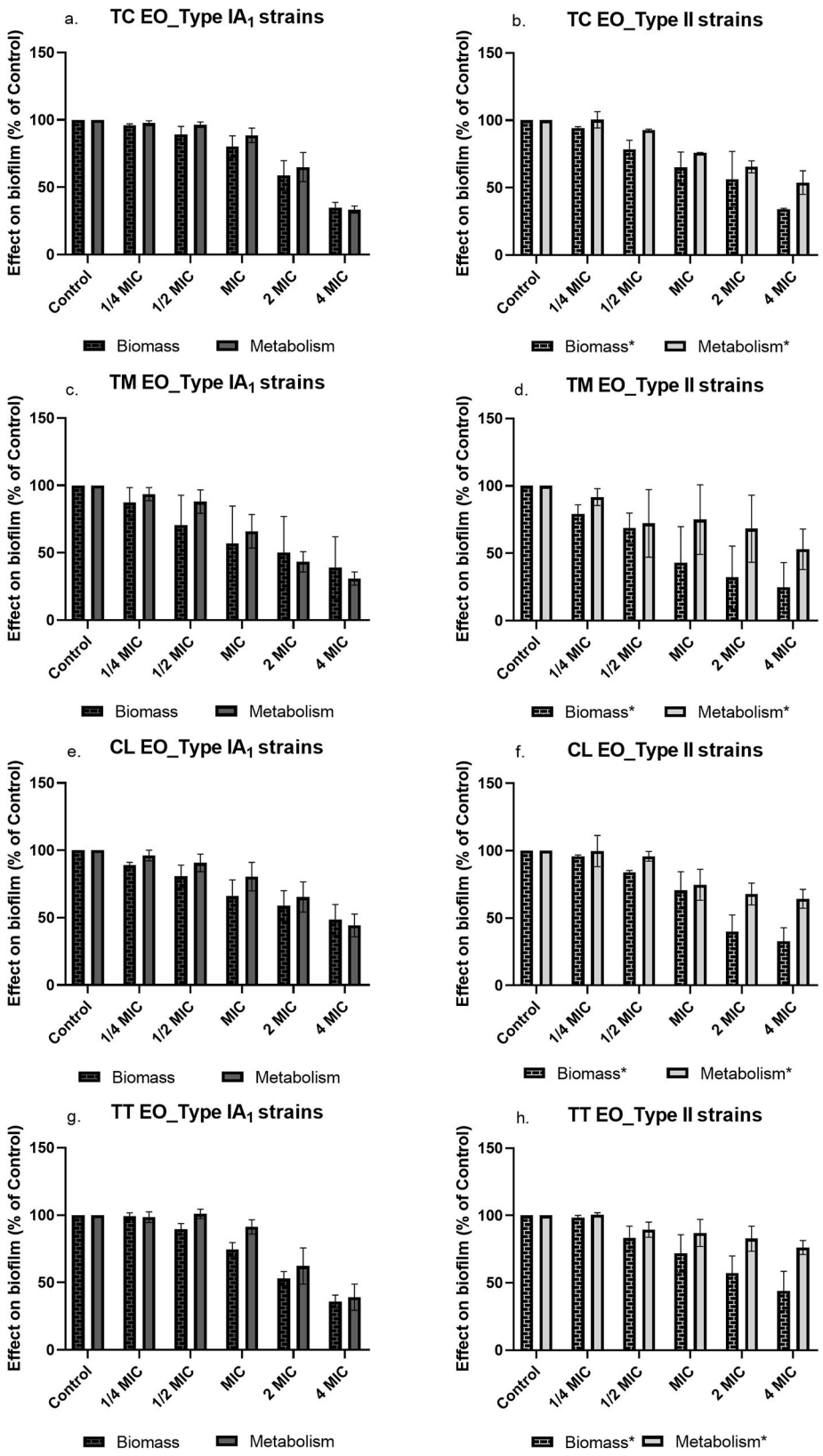

Fig. 8. Effect of the different EOs, specifically TC (a. and b.), TM (c. and d.), CL (e. and f.) and TT (g. and h.) in the impairment of biofilm biomass and on biofilm metabolic activity of *C. acnes* strains grouped by different phylotypes. Each EO was tested in concentrations ranging from one-fourth, half, once, twice to four times the planktonic MIC for each strain. Results are presented as a percentage of biofilm biomass or metabolic activity (mean ± SEM) for all strains grouped by phylotype. * Represents an overall statistically significant difference ($p < 0.05$) between results for metabolic activity and biofilm biomass, as determined by ordinary two-way ANOVA.

action of EOs is complex and their exact mechanism is still to be elucidated. Still, it has been primarily linked to their toxic effects on membrane structure and function [58,59]. Due to their lipophilic nature, EOs integrate into membranes, causing expansion, increased fluidity, and permeability, which disrupts cell walls and cytoplasmic membranes, leading to cell lysis and leakage of intracellular contents [64,65].

The different levels of efficacy found across EOs described to inherently related to their chemical composition and their synergistic interactions [59]. The presence of hydroxyl groups in EO's main components influences their antibacterial action, as oxygenated terpenes (terpenoids), are reported to present higher antimicrobial activity, than non-oxygenated ones [60]. While a hydrophobic skeleton is advantageous for penetration through the hydrophobic layers of the membrane, hydroxyl groups can act as a proton exchanger, retain terpenoids within the membrane layer, and affect various structural and dynamic membrane properties [61]. It has been also reported that acyclic compounds present higher antimicrobial activity compared with cyclic ones [62]. These structure-activity relations could account for the higher antimicrobial activity of TC EO, as it is composed mainly of geraniol, an acyclic monoterpenoid functionalized with a hydroxyl group. While similar logP values are present for geraniol, α-pinene and 1, 8-cineole (the major compounds for TC, CL and TM and TT, respectively), their polar surface area is quite different due to different functionalization groups, with geraniol presenting the highest value [63–65]. TC EO also presents thymol in its composition, a monoterpene phenol classified as one of the most active compounds in EOs [66]. Differences in chemical composition can also justify the difference in the antibacterial effect of the EOs, as α-pinene, the main component of CL EO, has been described as denoting bacteriostatic activity against Gram-positive bacteria [67]. To the best of our knowledge, apart from the results from our team, no reports on the antibacterial activity against *C. acnes* of TC, TM and CL EOs are available in the literature [19,31]. Regarding TT EO, several studies have investigated its antimicrobial activity against *C. acnes* strains, having reported MICs between 0.3 % (v/v) and 0.6 % (v/v), thus corroborating our results, as previously revised [15].

We found no statistically significant differences in EO activity among different phylogroups, presenting no selective efficacy. The small sample size may have contributed to this result. Still, TC showed a lower difference in MIC values between the two groups, affecting strains from both phylotypes similarly. Despite not being ideal, it was still a better result when compared with the other EOs. As the mechanisms described behind the antimicrobial activity of EOs are non-specific a major difference between strains was not expected [32]. Still, as differences in cell surface hydrophobicity are described among phylotypes, some differences regarding susceptibility to antimicrobials could still be present [68].

As discussed above, the strains' ability to form biofilms is an important factor related to treatment failure [5]. Based on this, we also evaluated the EOs' capacity to affect these structures, considering the effect on biofilm biomass and metabolic activity of sessile cells. When analyzing the strains individually, we found differences in the strain's response to the EOs, presenting a strain-dependent pattern. When strains were grouped by phylotype, no statistically significant differences were found regarding the overall effect on biofilm biomass and metabolic activity for phylotype IA_1 clinical strains, meaning that, in general, the EOs were equally effective in affecting the two outputs. Interestingly, strains from phylotype II required significantly higher concentrations to impair metabolic activity than biofilm biomass. They also required lower EO concentrations to impair biomass compared with phylotype IA_1 strains, considering the EC_{50} for biomass disruption. Despite presenting a similar trend when looking at MIC proportions, these refer to different concentrations, as each strain presents its individual MIC. As phylotype II strains present a less complex three-dimensional architecture, this could account for the lower resistance of their biofilm structure. Also, as phylotype II biofilms appear to present a high density of stacked cells, this could also justify the higher concentrations required to impair the metabolic activity of the cells within the biofilm. Still, other factors could be involved, as sessile cells present different growth rates and genetic and metabolic differences from planktonic cells [10,69]. When comparing the four EOs, TC EO proved to be the most effective, similarly reducing biomass and metabolic activity with lower concentrations, in all phylotype IA_1 clinical strains. EO composition can also account for their different levels of anti-biofilm activity. It has been reported that more hydrophilic EO' compounds show a higher anti-biofilm activity as the hydrophilic moieties can penetrate through the extracellular matrix, while the hydrophobic ones enable to permeate the bacterial membranes [70]. Again, these structure-activity hypotheses could account for the higher anti-biofilm activity of TC EO.

Considering the overall higher activity of TC EO, we propose that this EO could be positioned in the market similarly to TT EO. TT EO is an ingredient in many over-the-counter products for acne management and is also included in many cosmetic products targeting acneic skin, as a bioactive ingredient [71]. Thus, TC EO could be applied as a bioactive ingredient in cosmetic formulations targeting comodogenesis, as targeting this stage could prevent the progression to inflammatory lesions. As the variability in the compound concentration is one of the limitations of EO use, the creation of international standards for TC EO, defining minimum amounts of one or several compounds, would be of most relevance. The knowledge regarding the chemical composition is also relevant considering that EOs can serve as sources of allergens. Additional studies should be performed focusing on TC EO efficacy on other disease hallmarks and safety assessments in compliance with the European Regulation 1223/2009 on cosmetic products and on the opinions of the Scientific Committee on Consumer Safety.

5. Conclusion

Our results revealed a positive relation between phylotype IA_1 strains and the production of specific virulence traits such as biofilms and lipase, while others appeared strain-specific. They also revealed that phylotype IA_1 clinical strains present a more structurally complex biofilm than phylotype II strains. Regarding the activity of tested EOs, none presented a selective efficacy across phylotypes. Still, TC EO produced the lowest MIC values and a lower difference between MICs across the two phylogroups. TC was also more effective in impairing bacterial biofilms, a structure highly associated with treatment failure, equally affecting both biofilm biomass and metabolic activity of phylotype IA_1 clinical strains, at lower concentrations than CL, TM and TT EOs. These results highlight the anti-acne potential of TC EO, performing better than TT EO, a species with commercial uses for acne treatment. Even though TC did not present strain selectivity by itself, formulation strategies could be applied to maximize its effect on different phylotypes, based on differences in cell surface hydrophobicity or nutritional requirements. Further studies addressing the effect of TC EO in other hallmarks of the disease would also be of interest along with safety assessments to position it in the market as an alternative for acneic skin.

CRediT authorship contribution statement

Ana Sofia Oliveira: Writing – original draft, Methodology, Investigation, Formal analysis. **Carlos Gaspar:** Writing – review & editing, Validation, Methodology, Formal analysis. **Joana Rolo:** Writing – review & editing, Validation, Methodology. **Rita Palmeira-de-Oliveira:** Writing – review & editing, Methodology. **João Paulo Teixeira:** Writing – review & editing, Supervision. **José Martinez-de-Oliveira:** Writing – review & editing, Supervision, Conceptualization. **Ana Palmeira-de-Oliveira:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2024.107159.

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